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Hemoglobin adducts of ethylene oxide, propylene oxide, acrylonitrile and acrylamide–biomarkers in occupational and environmental medicine

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Abstract

In a chemical plant, ethylene oxide (EO) and propylene oxide (PO) were used for the production of surfactants for the textile industry. Within health supervision, we investigated the internal exposure of the workers using hemoglobin adducts as parameters of biochemical effects. The 95th percentile for N-2-hydroxyethylvaline (HEV) was 1280 pmol/g globin (= 29.4 μg/l blood) in blood from exposed workers compared with 100 pmol/g globin (or 2.3 μg/l) in controls. *N*-(*R*,*S*)-2-hydroxypropylvaline (HPV) both in workers and controls was below the detection limit (80 pmol/g globin or 2 μg/l). The levels of the adducts of acrylonitrile (ACN) and acrylamide (AA) were also determined, though they were mainly accounted for by smoking and diet. Median values of N-2-cyanoethylvaline (CEV) were below 4 pmol/g globin (or 0.1 μg/l) in non-smokers ($n = 24$) and 131 pmol/g globin (or 3.3 μg/l) in smokers ($n = 38$). Median values of N-2-carbamoylethylvaline (AAV) were 22 pmol/g globin (or 0.6 μg/l) in non-smokers compared with 89 pmol/g globin (or 2.4 μg/l) in smokers. Correlations were found between smoking habits and adduct levels of CEV and AAV.
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1. Introduction

Acrylamide (AA), acrylonitrile (ACN), ethylene oxide (EO) and propylene oxide (PO) are industrially important chemicals with various applications, e.g. the production of glycol ethers, non-ionic surfactants and polyacrylamides. Due to their high reactivity, these substances are able to bind to DNA molecules, or other macromolecular targets. Therefore, EO has been classified as a human carcinogen (Group 1) by the International Agency for Research on Cancer, AA has been rated as probably carcinogenic to humans (Group 2A), whereas ACN and PO are classified as possible human carcinogens (Group 2B) (IARC, 1994a,b,c, 1999). They are, moreover, constituents of tobacco smoke or, like AA, may be formed during the cooking process and, therefore, ingested in the diet. So, these substances are of great importance both in occupational and environmen-

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tal medicine. Hemoglobin adducts of these chemicals are valuable parameters of internal exposure and biochemical effects, which make it possible to estimate the individual health risk.

In our laboratory, we have set up an analytical method for the determination of globin adducts of AA (N-2-carbamoylethylvaline, AAV), ACN (N-2-cyanoethylvaline, CEV), EO (N-2-hydroxyethylvaline, HEV) and PO (N-(*R*,*S*)-2-hydroxypropylvaline, HPV) within the same analytical run. In order to determine the hemoglobin adduct levels of these alkylating substances in exposed workers, as well as the normal background in the general population, we investigated workers with occupational exposure to EO and PO and a control group.

2. Materials and methods

2.1. Subjects

A total of 62 employees (38 smokers, 24 non-smokers) of a chemical plant, occupied with the production of surfactants for the textile industry, took part in this study. The age of the workers ranged from 19 to 59 years with a median age of 35 years. All of the employees stated that they wore protective gloves during work. Data was obtained by questionnaire, with particular reference to smoking habits. The control group consisted of ten non-exposed persons (two smokers, eight non-smokers) from our laboratory. The median age of the control group was 30 years with a range of 27–59 years.

2.2. Chemicals

All chemicals used were of the highest analytical grade available and were purchased from Merck (Darmstadt, Germany), except for formamide. Formamide ultrapure was obtained from United States Biochemical (Cleveland, USA).

The dipeptide standards for the quantification of adducts: N-2-hydroxyethylvaline-leucine-anilide, N-(*R*,*S*)-2-hydroxypropylvaline-leucine-anilide, N-2-cyanoethylvaline-leucine-anilide, N-2-carbamoylethylvaline-leucine-anilide and N-2-ethoxyethylvaline-alanine-anilide as internal standard, were obtained from Bachem Biochemica (Heidelberg, Germany).

Pentafluorophenylisothiocyanate (PFPITC) was purchased from Fluka (Buchs, Suisse).

2.3. Blood sampling and isolation of globin

Biological adduct monitoring was conducted according to the method recommended by the Deutsche Forschungsgemeinschaft (van Sittert, 1996). Briefly, erythrocytes were isolated from 5 ml of whole blood by centrifugation (10 min, 800 g). The erythrocytes were washed three times with 5 ml of 0.9% NaCl solution. Hemolysis was achieved by addition of 5 ml of doubledistilled water and subsequent freezing at −18 °C. For the isolation of globin, 2 ml of hemolysate were added to 12 ml 50 mM hydrochloric acid in 2-propanol. The samples were shaken vigorously and centrifuged at 3500 × g for 10 min. Eight milliliters of ethyl acetate were slowly added to the supernatant and the solution was kept at +4 °C for at least 2 h. After centrifugation, the precipitated globin was washed two times with 5 ml ethyl acetate and 5 ml *n*-hexane and finally dried under vacuum overnight.

2.4. Determination of alkylated valine (modified Edman procedure)

One hundred milligrams of globin were dissolved in 3 ml formamide, 30 μl NaOH (1 M), 100 μl of a solution of the internal standard (N-2-EtOEt-Val-Ala-anilide, 1 mg/l in EtOH) and 15 μl of PFPITC were added. The samples were rotated overnight, at room temperature, and finally heated at 45 °C for 90 min in a water bath. The resulting pentafluorophenyl thiohydantoine derivatives of the N-terminal valine were extracted twice with 3 ml diethyl ether. The combined organic phases were evaporated to dryness under a gentle stream of nitrogen. The residue was then dissolved in 1.5 ml toluene and washed with 2 ml double distilled water and subsequently with 2 ml of a freshly prepared solution of Na_2CO_3 (0.1 M). Finally, the toluene phase was evaporated to dryness under nitrogen and re-dis-

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Table 1
Retention times and detected masses of pentafluorophenylthiohydantoin derivatives of analytes

Analyte	R (t)	Detected masses
N-2-hydroxyethylvaline (HEV)	20.33	308, 350
N-(R,S)-2-hydroxypropylvaline (HPV)	18.97	382, 364, 322
	19.27	382, 364, 322
N-2-cyanoethylvaline (CEV)	20.94	335, 377
N-2-carbamoylethylvaline (AAV)	26.18	395, 378, 363

Quantifier ions are underlined.

solved in 30 μl of toluene. One microliter was then analyzed by GC/MS in EI-Mode.

Calibration standards were prepared by spiking pooled globin of the control group with a solution of the dipeptide standards in the range of 0–7500 pmol/g globin (= 170 μg/l blood). The correlation coefficients of the resulting calibration curves were higher than $R^2 = 0.987$ for all analytes investigated. The limit of detection was 9 pmol/g globin (= 0.2 μg/l blood) for HEV, 80 pmol/g globin (= 2.0 μg/l blood) for HPV, 4 pmol/g globin (= 0.1 μg/l blood) for CEV and 11 pmol/g globin (= 0.3 μg/l blood) for AAV.

For quality control purposes, control globin of a smoker was included in each analytical series. The precision between series was 12.5% for HEV ($n = 6$, mean conc. 148 pmol/g globin). The between-series precision for CEV and AAV was found to be 8.3% ($n = 6$, mean conc. 168 pmol/g globin) and 13.7% ($n = 6$, mean conc. 89 pmol/g globin), respectively.

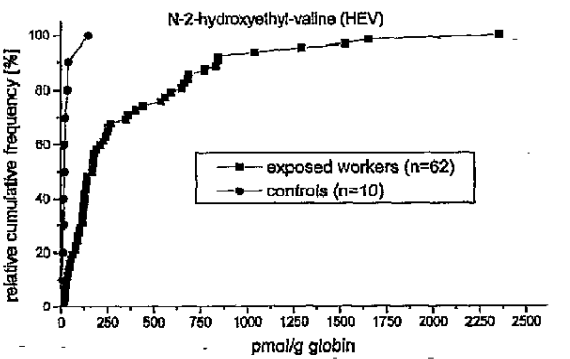

Fig. 1. Relative cumulative frequency for HEV in blood of exposed workers and controls.

2.5. GC/MS analysis

Analysis was carried out using a Hewlett–Packard HP G 1800A GCD-System gas chromatograph, equipped with a Hewlett–Packard HP 7673 autosampler and a split/splitless injector, operating in splitless mode. The inlet purge off time was 1 min. The operating temperature of the injector was 280 °C. Chromatographic separation was performed using a DB-17 HT capillary column (crosslinked (50%-Phenyl)-methylpolysiloxane, 30 m × 0.25 mm I.D., 0.15 μm film thickness, J&W Scientific, Folsom, CA, USA). Helium 5.0 was used as the carrier gas at a

Table 2
Results of biochemical effect monitoring

		HEV (pmol/g globin)	HPV (pmol/g globin)	CEV (pmol/g globin)	AAV (pmol/g globin)
	LOD	9	80	4	11
Controls ($n = 10$)	median	17	Not detectable	8	28
	95th percentile	100		112	71
	range	<9–150		<4–165	16–90
Exp. workers ($n = 62$)	median	168	Not detectable	62	63
	95th percentile	1280		237	144
	range	16–2353		<4–256	<11–294

LOD, limit of detection; HEV, N-2-hydroxyethylvaline; HPV, N-(*R,S*)-2-hydroxypropylvaline; CEV, N-2-cyanoethylvaline; AAV, N-2-carbamoylethylvaline.

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constant flow of 1.25 ml/min. The initial column temperature of 90 °C was held for 1 min, then raised at a rate of 25 °C/min to 120 °C. It was then raised at a rate of 5 °C/min to 240 °C and finally raised at 25 °C/min to 280 °C, where it remained for 20 min.

For mass spectrometric detection of the derivatives, a Hewlett–Packard HP MSD 5971 Series mass spectrometer, fitted with a quadrupole mass filter, was used in electron impact (EI) mode. EI mass spectra of the pentafluorphenylthiohydantoin derivatives were obtained at an energy level of 70 eV and the electron multiplier voltage was 2600 V (+600 rel.). The transfer line temperature was maintained at 300 °C. The retention times and detected masses for the pentafluorphenylthiohydantoin derivatives of the adducts under the described conditions are summarized in Table 1.

3. Results and discussion

The results of our study are summarized in Table 2. A significant difference was found between the workers and the control group for the parameter HEV, which is due to the exposure to EO in the plant. This is illustrated in the relative cumulative frequency for HEV shown in Fig. 1.

However, the concentrations of HEV found in the blood of the workers did not exceed the value of the German exposure equivalent for EO (90 μg/l blood = 3913 pmol/g globin), which has been evaluated by the Deutsche Forschungsgemeinschaft (DFG, 2001). Consequently, the exposure to this chemical can be considered as comparatively low.

No HPV could be identified neither in exposed workers nor in the controls. This may be due to the higher limit of detection for this parameter (LOD, 80 pmol/g globin), which is a consequence of the racemic separation of the enantiomers in two different peaks (see also Table 1) as well as an increased analytical background. Moreover, the rate of adduct formation has been reported to be five times lower for PO than for EO under comparable conditions (Boogaard et al., 1999).

The differences found for the adducts of ACN, CEV and AA, AAV are caused exclusively by the different smoking habits of the subjects. This can also be seen in Table 3, where the subject population has been subdivided into smokers and non-smokers. As this factor formed part of the questionnaire, we could establish correlations between self-reported daily consumption of cigarettes and the concentration of adducts determined in blood. These correlations are shown in Fig. 2.

Both parameters correlate well with the reported smoking habits, especially CEV (see Fig. 2A). According to our results, the daily consumption of one cigarette raises the adduct level of CEV for about 6.1 pmol/g globin. This is in good agreement with recent results of Fennell et al.

Table 3
Comparison between smoking and non-smoking workers

		HEV (pmol/g globin)	HPV (pmol/g globin)	CEV (pmol/g globin)	AAV (pmol/g globin)
	LOD	9	80	4	11
Non-smokers ($n = 24$)	Median	[redacted]	Not detectable	[redacted]	[redacted]
	95th percentile	988		14	46
	Range	16–2353		<4–71	<11–50
Smokers ($n = 38$)	Median	[redacted]	Not detectable	[redacted]	[redacted]
	95th percentile	1328		241	167
	Range	27–1653		12–256	16–294

LOD, limit of detection; HEV, N-2-hydroxyethylvaline; HPV, N-(*R*,*S*)-2-hydroxypropylvaline; CEV, N-2-cyanoethylvaline; AAV, N-2-carbamoylethylvaline.

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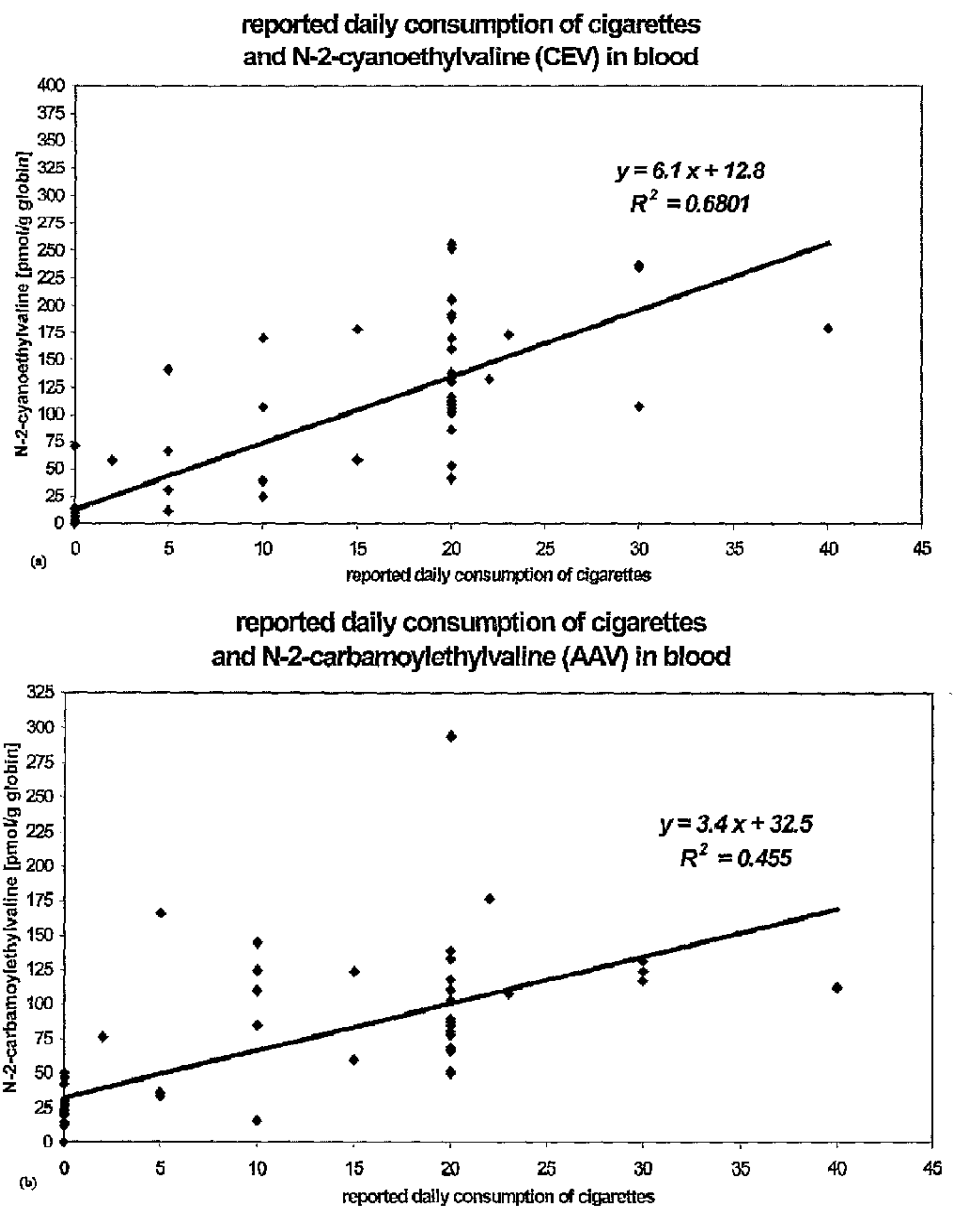

Fig. 2. (A) Correlations between daily consumption of cigarettes and adducts of ACN (CEV) determined in blood. (B) Correlation between daily consumption of cigarettes and adducts of AA (AAV) determined in blood.

(2000) who found an increase of 8.5 pmol/g globin per daily cigarette for this adduct.

The blood level of AAV is also closely connected with personal smoking habits (see Fig. 2B). Furthermore, this adduct could also be determined in the blood of the non-smoking general population with median values of 22 pmol/g globin (= 0.6 μg/l blood), indicating that other factors, such as diet, also contribute to the formation of this adduct (see also Table 2). Similar

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results have been described by Bergmark (Bergmark, 1997).

In addition, Tareke et al. have shown a significant increase of this adduct in the blood of rats fed with fried diet, compared with animals with normal diet, and even identified AA in the fried animal food (Tareke et al., 2000).

As AA may be formed in the cooking process, it is a very likely assumption that the background exposure of the non-smoking general population to AA, ascertained in this study, may also be caused by dietary intake.

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